

# Prospects & Overviews

## One-way trip: Influenza virus' adaptation to gallinaceous poultry may limit its pandemic potential

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We hypothesise that some influenza virus adaptations to poultry may explain why the barrier for human-to-human transmission is not easily overcome once the virus has crossed from wild birds to chickens. Since the cluster of human infections with H5N1 influenza in Hong Kong in 1997, chickens have been recognized as the major source of avian influenza virus infection in humans. Although often severe, these infections have been limited in their subsequent human-to-human transmission, and the feared H5N1 pandemic has not yet occurred. Here we examine virus adaptations selected for during replication in chickens and other gallinaceous poultry. These include altered receptor binding and increased pH of fusion of the haemagglutinin as well as stalk deletions of the neuraminidase protein. This knowledge could aid the delivery of vaccines and increase our ability to prioritize research efforts on those viruses from the diverse array of avian influenza viruses that have greatest human pandemic potential.

### Keywords:

■ H5N1; H7N9; influenza; pandemic; poultry

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### Abbreviations:

**HA**, haemagglutinin; **HPAI**, highly pathogenic avian influenza; **URT**, upper respiratory tract.

### Introduction

Avian influenza viruses do not readily infect humans because of the host range barriers that restrict them at a number of stages of their replication and transmission cycle [1]. Wild aquatic birds are the natural hosts for many antigenically distinct subtypes of influenza, and they occasionally pass their viruses to domesticated birds, where they may become endemic [2, 3]. During circulation in gallinaceous poultry (including chickens, turkeys, and quail; hereafter referred to as 'poultry'), influenza viruses undergo genetic adaptation, which sometimes results in greatly enhanced pathogenicity. The best described poultry adaptation is the acquisition of a novel sequence in the haemagglutinin (HA) gene, at the site where the precursor HA0 protein is cleaved by a host specific protease into its two components HA1 and HA2. The insertion of several basic amino acids at this site facilitates cleavage by proteases – such as furin – that are expressed in a wider range of tissues, and are often present inside cells [4–8]. This broadens the tissue tropism of the virus, resulting in dissemination throughout the body, and often rapid death of the infected bird. So far this type of adaptation to poultry has only naturally occurred in the H5 or H7 subtypes, resulting in what is termed a highly pathogenic avian influenza virus (HPAI). HPAI viruses do not seem to emerge in the natural wild waterfowl host, but if transmitted back to aquatic birds, the motif can be maintained. In aquatic birds, HPAI infections vary in outcome but sometimes result in large die offs such as the 2005 H5N1 outbreak at Lake Qinghai in China, which killed 6,000 migratory birds [9]. The consequence for pathogenicity in mammals of this poultry adaptation is less clear. In mice infected with H5N1 virus, the multibasic site (mbs) in HA is a major determinant of pathogenicity, and in human H5N1 infections reports of virus outside of the respiratory tract suggest that the mbs has conferred extended tropism [10, 11]. However, in nonhuman primates the mbs had less effect on the HP phenotype, and addition of mbs to other subtypes of virus does not always result in high pathogenicity, even in poultry [12, 13]. Whether an mbs is selected against during normal transmission between human or wild waterfowl hosts

is not clear, but this would be one explanation for the lack of such viruses emerging in non-poultry hosts. Exposure of humans to poultry is a more likely route for acquisition of an avian influenza virus than exposure to wild waterfowl. Therefore understanding the consequence for human infectivity of poultry adaptation of avian influenza viruses is crucial. In recent years, zoonoses following exposure of humans to poultry infected with avian influenza viruses have been documented, including more than 667 people infected with HPAI H5N1 virus [14], 450 people affected by HPAI H7N7 after a poultry outbreak in the Netherlands in 2003 [15], and several infections with avian viruses of the H9N2 [16–19], H6N1 [20], and H10N8 [21] subtypes in China, Taiwan, or Hong Kong. In spring 2013, a novel H7N9 virus infected at least 135 people in Eastern China, killing 44 of them [22]. In winter 2014 the virus re-emerged with a similarly high fatality rate, bringing the total number of confirmed cases to 450, including 165 deaths [23]. Chickens are considered to be the source of infection for most of these human cases. Unlike H5N1 and some other H7 viruses, the new H7N9 virus has not (yet) acquired a multi-basic HA cleavage site, and in fact it causes mild clinical signs in the infected chickens making it hard to detect and control in wet poultry markets [24]. Yet like H5N1, this avian influenza virus causes severe disease in most people whom it infects, but does not pass readily between them. A great deal of concern has been expressed in the past decade about whether avian influenza viruses will mutate to acquire increased transmissibility between humans. Lack of efficient human-to-human transmission is what currently spares us from what might otherwise be a devastating pandemic.

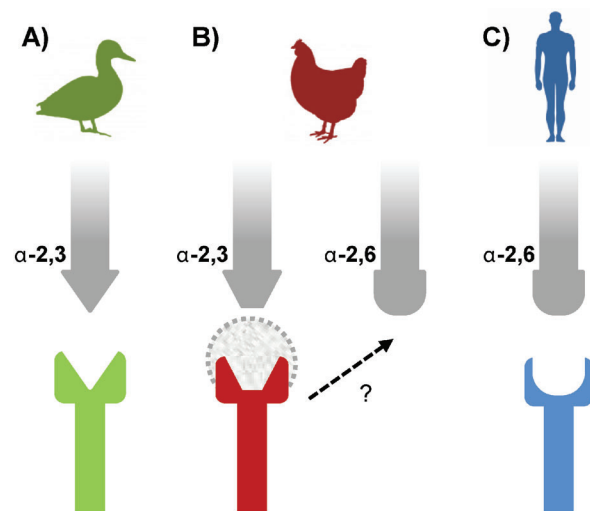
Transmission of influenza virus between humans is thought to occur by inhalation of respiratory droplets or aerosols containing infectious virus, or through transfer of virus from contaminated surfaces [25–27]. In either case, the virus particles must be shed in adequate amounts, retain sufficient infectivity as they traverse the physical gap between one host and another, and initiate infection in the new host with a small dose. Avian influenza viruses may be compromised at any of these transmission stages and need to acquire adaptive changes before they can sustain efficient circulation in humans. Here we describe three different adaptive changes that are associated with poultry-adapted influenza viruses: changes to receptor binding by HA, changes to stability of HA and changes to the stalk length of NA. We argue that some or all of these adaptations may drive virus evolution in a direction away from transmissibility between humans.

### Adaptation to poultry selects for mutations in haemagglutinin that affect receptor binding

It is widely accepted that efficient virus binding to the specific types of host cell receptors that are abundant in the upper respiratory tract (URT) of humans is a prerequisite for human transmission. Most natural strains of avian influenza virus do not bind well enough to the human receptors to be infectious at the low doses that might reach the target cells of the next host [28]. The HA is the viral attachment protein binding

sialic acid sugars, SA, that act as receptors on cell surfaces. Although HA binds SA with relatively low affinity, the HA is expressed in hundreds of trimerized copies adorning the virus particle, thereby increasing the overall avidity. Relatively small differences in affinity conferred by single or double amino acid mutations in the HA receptor-binding site transform into larger avidity changes that can shift the preference of the HA from one SA type to another [29]. Mutations within the HA receptor binding site that shift receptor binding preference away from the  $\alpha$ 2,3-linked sialic acids (SA) – which are more predominant in birds – to the  $\alpha$ 2,6-linked SA abundant in the URT of humans, were present in the H1, H2, and H3 HA subtype viruses that sparked the influenza pandemics of the 20th century [30–33] (Fig. 1). Mutations that alter the receptor-binding specificity affect transmission between ferrets. The ferret is a widely used animal model of human influenza, justified by its similar distribution of SAs to the human URT, and by similar clinical signs when infected with influenza [34–36]. We showed that changing two amino acids in the receptor-binding site of a human H3N2 influenza virus to those found in avian viruses abrogated transmission, because the avianized virus could not initiate infection at low doses [37]. Others have reported similar findings with H1N1 and H2N2 viruses [38, 39].

The chicken glycome (repertoire of glycan sugars) differs from that of aquatic birds (Fig. 1). Both  $\alpha$ 2,6- and  $\alpha$ 2,3-linked SAs are present in the chicken nasal cavity, upper respiratory tract, and gut, whereas in ducks  $\alpha$ 2,3-linked SA predominates [40–43]. During poultry adaptation HA evolves different SA-binding properties [44], which may actually



**Figure 1.** Differences in the glycome of influenza hosts drive changes in influenza HA that alter receptor binding. **A:** Avian influenza HA binds to  $\alpha$ -2,3-SA glycans presented on a cone-like structure that predominate in the duck. **B:**  $\alpha$ -2,3- and  $\alpha$ -2,6-glycans are present in the chicken, but their topology may differ from that in ducks or humans. We hypothesize that influenza HA must adapt to bind to a differently presented  $\alpha$ -2,3-glycan, perhaps by also acquiring HA glycosylation, and/or to bind to the  $\alpha$ -2,6-glycans also present, the topology of which may differ from the  $\alpha$ -2,6-glycans in humans. **C:**  $\alpha$ -2,6-glycans presented on umbrella-like structures in the human respiratory tract require the HA receptor binding site to accommodate their more bulky topology.

enhance the ability of the virus to infect cells in the human airway. Strikingly, the HA gene of the 2013 H7N9 virus already carries a single amino acid change in the receptor binding pocket, Q226L, that enhances binding to  $\alpha 2,6$  SA [22, 45]. This may account for the higher infection rate of people exposed to infected poultry than has been previously seen during H7 outbreaks, but it clearly is not sufficient alone to mediate human-to-human transmission [46]. Indeed, H9N2 influenza viruses widespread in poultry in Asia for some years also carry this change, and sporadic human infections have been documented, though with no evidence of onwards human spread [16]. Some have postulated that the additional amino acid change of glycine to serine at residue 228 would be required for transition to binding to human receptors [30, 31, 46]. However, this has not naturally occurred either in the H7 or the H9 HA in poultry, thus suggesting that the presentation of SA is different between poultry and humans.

However, other adaptations to poultry that alter the HA:SA interaction may adversely affect the ability of that HA to support virus transmission in humans. In particular, acquisition of glycosylation on the HA head domain is a common evolutionary change in chicken-adapted avian influenza viruses that may, via steric hindrance, decrease the strength of binding between virus HA and cell surface SA [47, 48]. It is not clear what advantage is brought to the virus in poultry by decreasing receptor-binding affinity, but one explanation is that this adaptation may be required to balance other changes driven by the poultry host (see below and Fig. 3). In controversial gain of function studies carried out in two laboratories and published in 2012, HPAI H5N1 viruses were experimentally generated that acquired transmissibility in ferrets [49, 50]. Both viruses carried pre-emptive mutations that enhanced binding to  $\alpha 2,6$ -SA receptors and simultaneously decreased  $\alpha 2,3$ -SA binding [29, 51]. During ferret passage, both viruses acquired a further mutation in the HA head domain that resulted in the loss of glycosylation at a site close to the receptor-binding site. This is consistent with an earlier report that showed that the removal of this glycosylation site (158N), in addition to Q226L and G228S that affect SA specificity, enhanced replication of a live attenuated H5N1 vaccine virus in the ferret URT and increased its immunogenicity [52]. The loss of HA head glycosylation in ferret transmissible H5N1 viruses increased virus binding to both  $\alpha 2,6$ - and  $\alpha 2,3$ -SA receptors [53], and suggests that the HA:SA balance needs to be repaired in the mammalian host to support transmission. Thus, acquiring HA head glycosylation in poultry results in a virus that is less likely to be transmitted in mammals. However it should be noted that, as H5N1 virus continues to circulate widely amongst avian species, some natural isolates of H5N1 virus that already lack glycosylation at HA residues 158–160 have been reported in Egypt, demonstrating that this potential host range barrier is rather readily lost [54, 55].

Taken together these observations suggest that, during outbreaks of avian influenza viruses in poultry, HA sustains mutations that affect receptor binding, and some of these may sterically hinder the virus host interaction in ways presumably advantageous for poultry but deleterious for transmission between humans. Other mutations that enhance binding to human receptors are not on their own

sufficient to enable transformation into a human transmissible virus [53, 56].

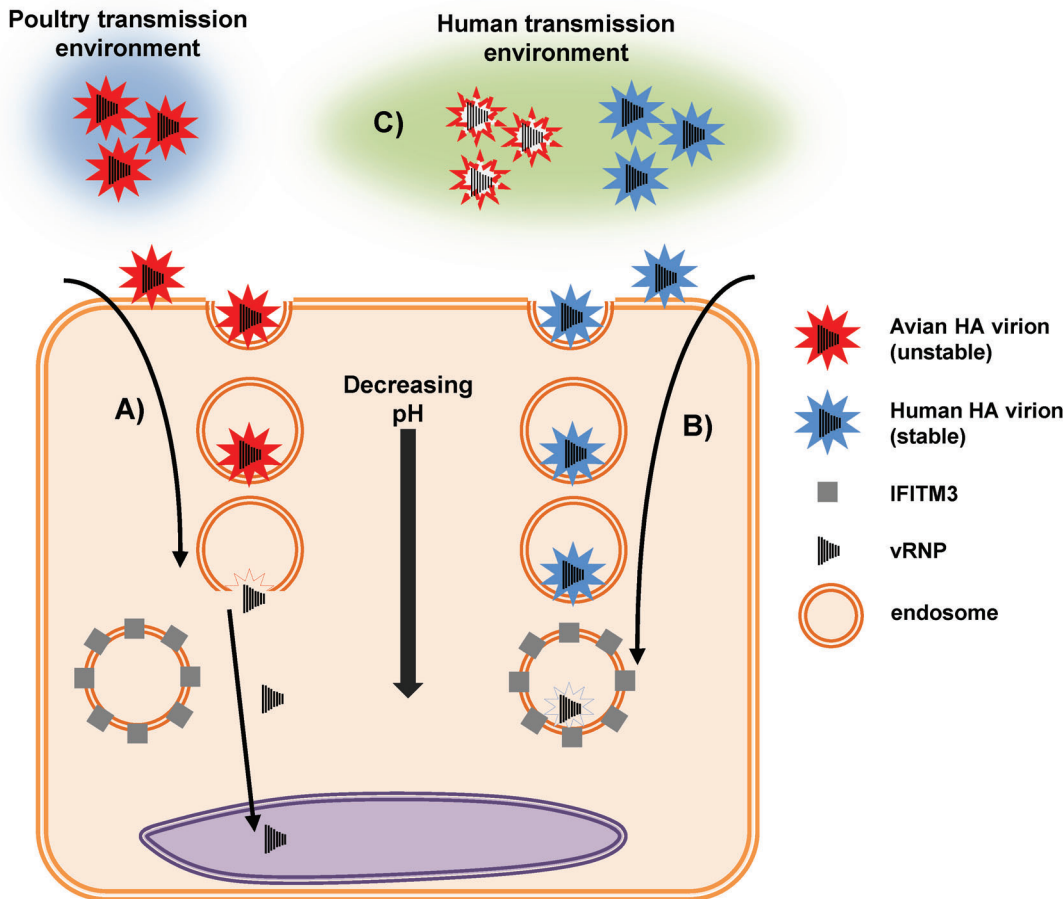
## The pH stability of HA affects both virulence and transmission

As well as mediating receptor binding, HA is the fusogenic protein of the virus. After HA binds SA receptors on the host cell surface, the virion enters the cells by endocytosis. Inside the acidic environment of the endosome, HA undergoes a conformational change [57, 58], whereby ionisable residues situated in the stalk region trigger fusion [8, 59–62]. The pH at which HA undergoes this change varies between different subtypes and strains of virus [63]. Interestingly, many of the HA proteins from poultry-adapted viruses have a higher (less acidic) pH for fusion than typical human adapted virus HAs, making them less pH stable. Indeed the pH instability of particular HA proteins from HPAI (previously known as Fowl plague viruses FPV) was noted many years ago [64, 65]. This may contribute to the HPAI phenotype, since more recently, Dubois et al. showed that a H5N1 virus with an unstable HA caused increased pathogenicity in the chicken host [66].

## Why might unstable HA proteins, as observed in poultry adapted viruses, be advantageous to the virus?

An advantage to the virus of possessing HA that fuses at relatively high pH is the opportunity to release its genome from the early endosome rather than waiting for further endosome maturation. This might give the virus a 'head start' to initiate replication before the host cell's innate response is activated. Another reason for early escape from the endosomal pathway is to avoid lysosomal degradation, and/or to evade the inhibitory effects of innate restriction factors such as interferon-induced transmembrane protein 3 (IFITM3) that reside in the late endosomes and inhibit viral fusion [67] (Fig. 2). In humans and mice, IFITM3 has a crucial role in limiting influenza-induced morbidity and mortality [68].

Under circumstances of virus propagation where transmission is not required, the advantages conferred by an unstable HA are evident. Serial passage in either cell culture [69] or in mice [70, 71] has been shown to select viruses with HAs that fuse at higher pH, supporting the notion that this phenotype confers a selective advantage under laboratory conditions. In addition, a highly virulent PR8 variant carries an HA mutation that elevates fusion pH and contributes to its virulence in mice [72]. Although recent reports have identified IFITM3 orthologs capable of restricting influenza in chickens [68] and in pigs (Benfield et al, submitted), an intriguing hypothesis is that species-specific differences in endosomal pH or in the potency or location of IFITM3 (or other IFITM) proteins may differentially restrict influenza viruses. Whether such factors underlie the emerging importance of HA acid stability to virus cross species transmission (see below) is an area for future study.



**Figure 2.** Trade-off between intracellular and extracellular effects of HA stability. **A:** Poultry-adapted HA virion fuses at a higher pH in the early endosome and so is less susceptible to restriction by IFITM3, which resides in the late endosomes. vRNPs leave endosomes rapidly and traffic to the nucleus for replication. **B:** Human HA virion can only fuse at the lower pH in the late endosome and so is more susceptible to restriction by IFITM3. IFITM3 prevents vRNPs from escaping the endosome to initiate replication. **C:** The unstable poultry-adapted HA is more susceptible to deactivation in the human transmission environment.

### The cost of instability

However, triggering of HA fusion activity at higher pH equates to virion instability. This comes at a cost during transmission events, and this cost will be greater when hosts are more dispersed and successful transmission requires environmental longevity or resilience. For each particular host species and transmission mode, the virus must strike a balance between stability and uncoating. In the primordial influenza reservoir, the route for influenza transmission between natural water-fowl hosts is through shared water. Here host population is less dense, and environmental survival might be a more stringent driver than early endosome release. Indeed, the work of Stallknecht demonstrates that environmental stability is one driver of avian influenza virus evolution, and modelling by the same group implies that the route of transmission will influence the pressure on the virus to retain temperature

stability [73, 74]. Moreover, Reed et al. showed that survival of avian influenza viruses carrying HA destabilizing mutations in water was compromised, and transmission between ducks was less efficient. On the other hand mutations that overstabilized HA were also deleterious in the anseriforme (duck) host because they attenuated the virus, presumably by delaying fusion [75]. For terrestrial birds, including poultry, transmission might occur by the fecal-oral or respiratory routes. In the crowded poultry house, a virus particle may not need to survive long in the environment before reaching a new host: thus benefits of early fusion might outweigh the costs of instability. This balance between survival in the environment and efficiency of uncoating inside the cell may be a concept that applies to other viruses. Pfeiffer's work on poliovirus also shows that mutations that decrease stability compromise environmental survival and transmission although they have little effect on virus replication *in vitro* [76].

### Why should HA stability be particularly important for respiratory droplet transmission between mammals?

Efforts to understand the mutations required for human transmission of H5N1 avian influenza viruses reached a controversial climax in 2012 with the generation of two H5N1 viruses with increased transmissibility between ferrets,



selected using a combination of reverse and traditional genetics [49, 50].

The unexpected and consistent result from the H5 ferret transmission studies was the finding that the acquisition of airborne transmissibility required not only changes in HA receptor binding, but also mutations further down the HA protein, in the stalk region or on the trimer interface, T318I or H103Y. This discovery reiterated findings of a study published by Wan, Sorrell, and Perez some years earlier describing an H9N2 avian influenza virus that became ferret transmissible with the combination of receptor binding changes and an amino acid change in the HA stalk region, H192R [77, 78]. Both the T318I and H103Y mutations have been shown to increase HA stability (i.e. lower fusion pH) [49, 53], and although not yet proven, the location of the H9N2 H192R mutation suggests a similar function was required for adaptation of this virus.

During respiratory droplet transmission, incoming virus particles must deposit on the apical surface of the respiratory tract, having traversed the nasal mucosa. Interestingly, studies in humans have shown this environment as mildly acidic: measurements indicate a range between pH 5 and 8, with a mean pH of 6.3 [79, 80]. Thus an unmodified HPAI poultry H5 virus that lands in the ferret or human nose might be inactivated by the premature triggering of HA rearrangement before it can bind and enter its target cell. Put another way, the dose of incoming virus required to successfully initiate infection in a new host might be higher than can be achieved during natural transmission routes if the HA is prone to instability.

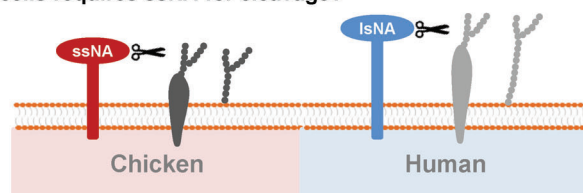
The pH at mucosal surfaces of other host species for influenza such as the chicken or pig has not to our knowledge been recorded. The circumstances of transmission – for example, crowded or disperse hosts – may be what primarily determines the balance between stability and virulence for influenza, rather than physiological differences between its hosts. Even in poultry, viruses that retain HA stability may transmit through the air more efficiently compared with isolates with less stable HA, as recently shown with a pair of H9N2 chicken viruses [74].

Following introduction of a novel virus to humans in a pandemic, transmissibility becomes increasingly important as the virus continues to circulate, but the number of susceptible naive hosts dwindles. After the initial emergence of the swine origin H1N1 pandemic virus in 2009, further adaptation was observed in subsequent pandemic waves during circulation in humans [81]. In HA, mutations that decreased the pH of fusion from 5.4 to 5.0 were acquired and maintained [82]. This increased infectivity in ferrets and may well have contributed to the ability of the virus to continue to circulate in subsequent years even in the face of accumulating human immunity [83].

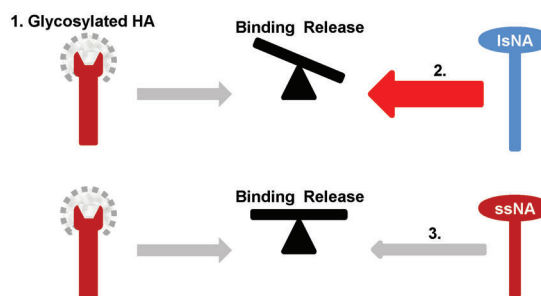
## Deletions in the neuraminidase stalk attenuate influenza virus in humans

A second way in which adaptation to poultry might restrict an influenza virus from becoming transmissible in humans maps to the neuraminidase gene.

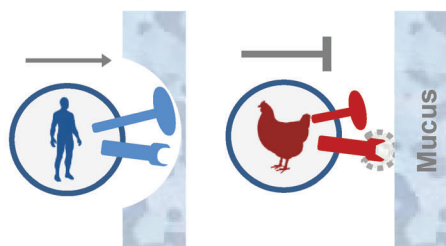
### A) Steric presentation of sialic acid substrates on chicken cells requires ssNA for cleavage?



### B) Glycosylation of HA affects sialic acid receptor binding affinity and HA:NA activity is rebalanced by ssNA?



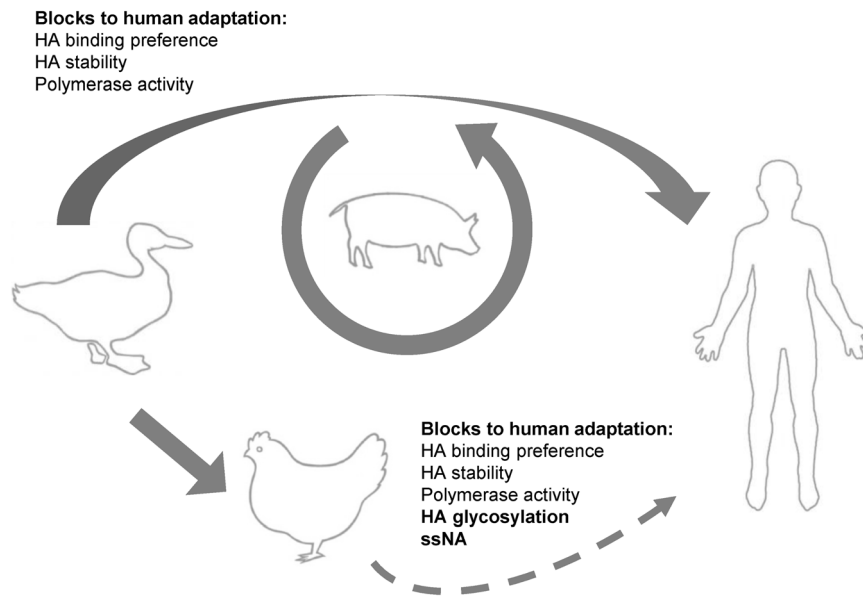
### C) ssNA blocked by human mucus barrier



**Figure 3.** Poultry adaptations of HA and NA restrict infection of the human respiratory tract. **A:** The presentation of chicken cell SA substrates may differ from that of duck and human cells, requiring the NA to truncate its stalk in order to efficiently cleave and release virions from the cell surface. **B:** 1. HA acquires glycosylation in poultry, reducing the binding affinity to SA. 2. With unadapted IsNA the cleavage of SA may outweigh the binding affinity of HA. 3. Truncation to ssNA reduces cleavage of cell surface SA and rebalances HA:NA activity. **C:** IsNA is sufficiently able to cleave SA substrates present in human mucus, allowing progression through this innate barrier to initiate infection at the respiratory epithelium. ssNA is unable to cleave these substrates and virus is therefore blocked by the human mucus barrier.

The neuraminidase (NA) presents a tetrameric sialidase enzyme atop a stalk that extends from the infected cell or virion particle surface. NA desialates glycans on the cell surface to aid virus release and on HA and NA glycoproteins to prevent virion aggregation. In addition, NA removes SA from molecules in mucus that otherwise act as decoy receptors and deter virus access to the epithelial cell surface [84, 85].

During adaptation to poultry from an aquatic bird source, as described above, the HA gene often acquires glycosylation that occludes the SA-binding site. Concomitantly the NA protein undergoes truncation of its stalk [86–89]. Stalk truncation reduces the height of the NA by the deletion of



**Figure 4.** Summary of the reduced pandemic potential of poultry-adapted influenza viruses compared with influenza viruses of aquatic bird origin. Mammalian host range barriers that are common to both poultry-adapted influenza viruses and those adapted to aquatic birds include HA-binding preference, HA stability, and polymerase activity. Adaptation to the poultry host often results in the accumulation of additional mutations including HA glycosylation and NA truncation, evolving the virus further away from adaptation to mammals (dotted arrow). In both cases, zoonotic transmission may occur, but onward human-to-human transmission does not follow in the absence of additional adaptations.

between 1 and 35 amino acids. Studies show that this does not affect the enzyme's capacity to cleave a soluble sialic acid substrate [90–92]. However, the truncation may sterically compromise cleavage of sialic acid on tethered substrates, such as those on HA of neighboring virus particles or cell surface receptors, leading to virion aggregation or lack of release from the infected cells. In addition, an inability to cleave decoy receptors in multivalent substrates such as mucus may result in poor access to the apical surface of respiratory cells. Why stalk truncation arises in poultry is not yet explained. It may be a rapid way for the virus to rebalance the HA-NA relationship after acquiring HA glycosylation (Fig. 3). Alternatively the NA truncation could drive the HA change, and the shorter stalk of NA may relate to a difference in the specific abundance or topological presentation of sialic acids in the target tissues in poultry. In experimental studies, it is clear that stalk truncation conferred increased virulence in chickens, and interestingly also in mice [91, 93]. However, studies in the ferret model suggest that, in these hosts, truncation of the NA stalk is deleterious for transmissibility, and there are no natural human viruses that bear truncation in NA [90, 94, 95].

When the HA from an H5N1 virus (which usually had a short stalk) was paired with NA from a human-adapted virus (long stalk) [96, 97], it increased replication in human cells and transmission in ferrets, reinforcing the concept that short stalk NA compromises replication in the mammalian respiratory tract. In one of the two papers describing respiratory droplet (RD) transmission of HPAI H5N1 virus in ferrets, the H5 HA gene had been combined with an otherwise human-adapted genetic backbone that encoded a NA with a full length stalk [49]. In the other paper H5N1 RD transmission was achieved with a short stalk NA, but in comparison with transmission dynamics of pandemic H1N1 2009 influenza virus, transmission efficacy was low [50].

We recently showed that in ferrets, an otherwise transmissible pH1N1 2009 virus lost the transmissible phenotype when it encoded a NA derived from an H5N1

virus naturally truncated by 20 amino acids, and this block was overcome simply by extending the NA stalk length back to that seen in human-adapted viruses [90].

In vitro studies suggested that mucus secreted from the respiratory epithelium acts as an important neutralizing barrier to influenza virus infectivity because it presents decoy sialic acid receptors [37, 85]. To counter this barrier the viral NA can desialate the mucus over time, hence allowing virus to penetrate to the apical surface of ciliated epithelium below. However, we showed that virus with a short stalk NA was compromised in its ability to overcome mucus inhibition, a deficit that was repaired by extending the stalk length [90]. This suggests that access of the enzyme head to the mucus substrate is impaired in chicken-adapted viruses with short NA stalks (Fig. 3). Whether chickens lack the same level of respiratory mucus or whether the types of sialic acid chains that adorn it differ from those in the mammalian respiratory tract is not clear, but this aspect may be worthy of future attention.

The N9 NA from the recently emerged H7N9 virus manifests a short five amino acid truncation, whereas the truncation of NAs from H5N1 viruses tends to be much longer, usually a 20 aa truncation [22, 98]. Therefore, whether the N9 NA truncation affects the ability of the virus to function in the human respiratory tract environment is not yet clear. This may present a further barrier that prevents efficient human transmission of the current H7N9 viruses.

### Implications of poultry adaptation in HA and NA for the failure of pre-Pandemic live attenuated influenza vaccine

Vaccination is the primary measure for control of an influenza outbreak. However, pandemic vaccines based on avian HAs such as H5 or H7 that have been tested in phase I trials have

yielded disappointing results. These inactivated vaccines require larger doses than for seasonal influenza vaccine and more than one immunization [99]. Live attenuated influenza vaccines with H5 or H7 HA so far have fared even worse [100]. Several phase I clinical trials were performed using LAIV with H5 or H7 HAs but an almost total lack of replication in the nose of recipients resulted in no seroconversion measured either by hemagglutination inhibition (HI) or neutralization. With hindsight this result is not too surprising: The basis of attenuation of the LAIV vaccines is that the core proteins of the virus are cold-adapted and temperature sensitive [101]. Thus LAIV only replicates in the cooler temperatures of the nose. However, as discussed above, viruses with avian HA and NA – particularly if derived from poultry adapted strains – are highly restricted in the human nose. Even when mutations were engineered into the H5 HA to increase  $\alpha$ 2,6-SA binding ability, the replication of H5 LAIV in ferrets was still low [52]. Egorov and co-workers found that engineering a mutation to the H5 HA that stabilized against low pH increased replication in the nose of mice, and enhanced the antibody response induced by an attenuated virus [102].

Engineering virus with H5 HA combined with a different (long stalk) NA increased mammalian cell replication and ferret nasal titres [96]. We recently combined these strategies by engineering receptor binding and pH stabilizing changes into a recombinant vaccine virus with H5 HA and long stalk N1 NA. We found that these changes led to increased viral shedding from the nose of infected ferrets, to the extent that contact transmission occurred, and that the mutations in H5 HA did not compromise the antigenicity of H5 [97].

The improved immunogenicity of LAIV over inactivated or subunit AI vaccines suggests fewer doses of a LAIV vaccine, each made with less virus, would be required; hence a greater proportion of the population could be vaccinated [103]. Moreover, increased pH stability and heat stability appear to go hand in hand, and this will increase the longevity of the vaccines produced [82]. While it can be argued that the H5 virus that acquired human transmissibility would necessarily come ready-made with these adaptive HA and NA mutations, if we wait for nature to generate the virus, we won't have time to test the feasibility and safety of such a LAIV approach in preclinical and clinical trials.

Thus we propose that LAIV HA stable H5 and H7 vaccines should be generated and tested under appropriate containment conditions for their ability to replicate in the URT of ferrets and then humans. We postulate that these modified LAIV will induce antibody and cellular immune responses and constitute a new pre-pandemic vaccine.

## Conclusions and outlook

In summary, adaptation of influenza A viruses in poultry selects for HAs with additional glycosylation sites that impair human receptor binding; it also selects for increased pH of fusion that results in environmental fragility, and NA stalk length truncation that impairs the virus' ability to overcome the mucus barrier (Figs. 1, 2, and 3).

These restrictions combine with the other well characterized host range restrictions of SA receptor specificity and inadequate polymerase function that also restrain avian influenza viruses from crossing the host barrier (Fig. 4). Taken together, the barriers that a poultry-adapted influenza virus has to overcome in order to contribute to a new pandemic are relatively high.

Nonetheless, one certainly cannot exclude that such barriers can be overcome, particularly bearing in mind the huge numbers of poultry and the close contact between humans and poultry in certain world regions. Therefore, it is important to use this new knowledge to improve our pandemic preparedness.

Surveillance for risk-assessing the threat from H7N9 [22], H10N8 [21], or any other emerging avian influenza virus might include an assessment of the pH stability of the HA and the ability of the NA to digest human respiratory mucus in addition to receptor binding characteristics as an early indicator of the likelihood of its transmission potential [104]. Gabbard et al. recently showed that the HA of the 2013 H7N9 virus displayed a high fusion pH typical of poultry viruses, and we suggest that this, combined with its NA stalk length truncation, may explain its lack of human transmission, despite the receptor-binding site mutation that it already carries [105].

## References

1. Cauldwell AV, Long JS, Moncorgé O, Barclay WS. 2014. Viral determinants of influenza A host range. *J Gen Virol* **95**: 1193–210.
2. Webster RG, Bean WJ, Gorman OT, Chambers TM, et al. 1992. Evolution and ecology of influenza A viruses. *Microbiol Rev* **56**: 152–79.
3. Alexander DJ. 2000. A review of avian influenza in different bird species. *Vet Microbiol* **74**: 3–13.
4. Rott R, Klenk HD, Nagai Y, Tashiro M. 1995. Influenza viruses, cell enzymes, and pathogenicity. *Am J Respir Crit Care Med* **152**: S16–9.
5. Bosch FX, Garten W, Klenk HD, Rott R. 1981. Proteolytic cleavage of influenza virus hemagglutinins: primary structure of the connecting peptide between HA1 and HA2 determines proteolytic cleavability and pathogenicity of avian influenza viruses. *Virology* **113**: 725–35.
6. Garten W, Bosch FX, Linder D, Rott R, et al. 1981. Proteolytic activation of the influenza virus hemagglutinin: The structure of the cleavage site and the enzymes involved in cleavage. *Virology* **115**: 361–74.
7. Perdue ML, Garcia M, Senne D, Fraire M. 1997. Virulence-associated sequence duplication at the hemagglutinin cleavage site of avian influenza viruses. *Virus Res* **49**: 173–86.
8. Chen J, Lee KH, Steinhauer DA, Stevens DJ, et al. 1998. Structure of the hemagglutinin precursor cleavage site, a determinant of influenza pathogenicity and the origin of the labile conformation. *Cell* **95**: 409–17.
9. Zhou JY, Shen HG, Chen HX, Tong GZ, et al. 2006. Characterization of a highly pathogenic H5N1 influenza virus derived from bar-headed geese in China. *J Gen Virol* **87**: 1823–33.
10. Hatta M, Gao P, Halfmann P, Kawaoka Y. 2001. Molecular basis for high virulence of Hong Kong H5N1 influenza A viruses. *Science* **293**: 1840–2.
11. De Jong MD, Simmons CP, Thanh TT, Hien VM, et al. 2006. Fatal outcome of human influenza A (H5N1) is associated with high viral load and hypercytokinemia. *Nat Med* **12**: 1203–7.
12. Schrauwen EJ, Fouchier RA. 2014. Host adaptation and transmission of influenza A viruses in mammals. *Emerg Microbes Infect* **3**: e9.
13. Suguitan AL, Matsuoka Y, Lau Y-F, Santos CP, et al. 2012. The multibasic cleavage site of the hemagglutinin of highly pathogenic A/Vietnam/1203/2004 (H5N1) avian influenza virus acts as a virulence factor in a host-specific manner in mammals. *J Virol* **86**: 2706–14.
14. World Health Organization. 2014. Cumulative number of confirmed human cases of avian influenza A(H5N1) reported to WHO. [http://www.who.int/influenza/human\\_animal\\_interface/H5N1\\_cumulative\\_table\\_archives/en/](http://www.who.int/influenza/human_animal_interface/H5N1_cumulative_table_archives/en/). Accessed 8th October 2014.



15. Koopmans M, Wilbrink B, Conyn M, Natrop G, et al. 2004. Transmission of H7N7 avian influenza A virus to human beings during a large outbreak in commercial poultry farms in the Netherlands. *Lancet* **363**: 587–93.
16. Lin YP, Shaw M, Gregory V, Cameron K, et al. 2000. Avian-to-human transmission of H9N2 subtype influenza A viruses: relationship between H9N2 and H5N1 human isolates. *Proc Natl Acad Sci USA* **97**: 9654–8.
17. Peiris M, Yuen KY, Leung CW, Chan KH, et al. 1999. Human infection with influenza H9N2. *Lancet* **354**: 916–7.
18. Butt KM, Smith GJD, Chen H, Zhang LJ, et al. 2005. Human infection with an avian H9N2 influenza A virus in Hong Kong in 2003. *J Clin Microbiol* **43**: 5760–7.
19. Cheng VCC, Chan JFW, Wen X, Wu WL, et al. 2011. Infection of immunocompromised patients by avian H9N2 influenza A virus. *J Infect* **62**: 394–9.
20. Wei S-H, Yang J-R, Wu H-S, Chang M-C, et al. 2013. Human infection with avian influenza A H6N1 virus: an epidemiological analysis. *Lancet Respir Med* **1**: 771–8.
21. Chen H, Yuan H, Gao R, Zhang J, et al. 2014. Clinical and epidemiological characteristics of a fatal case of avian influenza A H10N8 virus infection: a descriptive study. *Lancet* **383**: 714–21.
22. Gao R, Cao B, Hu Y, Feng Z, et al. 2013. Human infection with a novel avian-origin influenza A (H7N9) virus. *N Engl J Med* **368**: 1888–97.
23. World Health Organization. 2014. Avian influenza A(H7N9) virus. [http://www.who.int/influenza/human\\_animal\\_interface/influenza\\_h7n9/en/](http://www.who.int/influenza/human_animal_interface/influenza_h7n9/en/). Accessed 8th October 2014.
24. Li J, Yu X, Pu X, Xie L, et al. 2013. Environmental connections of novel avian-origin H7N9 influenza virus infection and virus adaptation to the human. *Sci China Life Sci* **56**: 485–92.
25. Cowling BJ, Ip DKM, Fang VJ, Suntarattiwong P, et al. 2013. Aerosol transmission is an important mode of influenza A virus spread. *Nat Commun* **4**: 1935.
26. Killingley B, Nguyen-Van-Tam J. 2013. Routes of influenza transmission. *Influenza Other Respi Viruses* **7**: 42–51.
27. Tellier R. 2006. Review of aerosol transmission of influenza A virus. *Emerg Infect Dis* **12**: 1657–62.
28. Matrosovich M, Stech J, Klenk HD. 2009. Influenza receptors, polymerase and host range. *Rev Sci Tech* **28**: 203–17.
29. Xiong X, Coombs PJ, Martin SR, Liu J, et al. 2013. Receptor binding by a ferret-transmissible H5 avian influenza virus. *Nature* **497**: 392–6.
30. Matrosovich M, Tuzikov A, Bovin N, Gambaryan A, et al. 2000. Early alterations of the receptor-binding properties of H1, H2, and H3 avian influenza virus hemagglutinins after their introduction into mammals. *J Virol* **74**: 8502–12.
31. Connor RJ, Kawaoka Y, Webster RG, Paulson JC. 1994. Receptor specificity in human, avian, and equine H2 and H3 influenza virus isolates. *Virology* **205**: 17–23.
32. Vines A, Wells K, Matrosovich M, Castrucci MR, et al. 1998. The role of influenza A virus hemagglutinin residues 226 and 228 in receptor specificity and host range restriction. *J Virol* **72**: 7626–31.
33. Rogers GN, Paulson JC. 1983. Receptor determinants of human and animal influenza virus isolates: differences in receptor specificity of the H3 hemagglutinin based on species of origin. *Virology* **127**: 361–73.
34. Belser JA, Katz JM, Tumpey TM. 2011. The ferret as a model organism to study influenza A virus infection. *Dis Model Mech* **4**: 575–9.
35. O'Donnell CD, Subbarao K. 2011. The contribution of animal models to the understanding of the host range and virulence of influenza A viruses. *Microbes Infect* **13**: 502–15.
36. Jia N, Barclay WS, Roberts K, Yen H-L, et al. 2014. Glycomic characterisation of respiratory tract tissues of ferrets: implications for its use in influenza virus infection studies. *J Biol Chem* **289**: 28489–504.
37. Roberts KL, Shelton H, Scull M, Pickles R, et al. 2011. Lack of transmission of a human influenza virus with avian receptor specificity between ferrets is not due to decreased virus shedding but rather a lower infectivity in vivo. *J Gen Virol* **92**: 1822–31.
38. Tumpey TM, Maines TR, Van Hoeven N, Glaser L, et al. 2007. A two-amino acid change in the hemagglutinin of the 1918 influenza virus abolishes transmission. *Science* **315**: 655–9.
39. Pappas C, Viswanathan K, Chandrasekaran A, Raman R, et al. 2010. Receptor specificity and transmission of H2N2 subtype viruses isolated from the pandemic of 1957. *PLoS One* **5**: e11158.
40. Kuchipudi SV, Nelli R, White GA, Bain M, et al. 2009. Differences in influenza virus receptors in chickens and ducks: Implications for interspecies transmission. *J Mol Genet Med* **3**: 143–51.
41. Costa T, Chaves AJ, Valle R, Darji A, et al. 2012. Distribution patterns of influenza virus receptors and viral attachment patterns in the respiratory and intestinal tracts of seven avian species. *Vet Res* **43**: 28.
42. Gambaryan A, Webster R, Matrosovich M. 2002. Differences between influenza virus receptors on target cells of duck and chicken. *Arch Virol* **147**: 1197–208.
43. Wan H, Perez DR. 2006. Quail carry sialic acid receptors compatible with binding of avian and human influenza viruses. *Virology* **346**: 278–86.
44. Gambaryan AS, Tuzikov AB, Pazynina GV, Desheva JA, et al. 2008. 6-sulfo sialyl Lewis X is the common receptor determinant recognized by H5, H6, H7 and H9 influenza viruses of terrestrial poultry. *Virol J* **5**: 85.
45. Shi Y, Zhang W, Wang F, Qi J, et al. 2013. Structures and receptor binding of hemagglutinins from human-infecting H7N9 influenza viruses. *Science* **342**: 243–7.
46. Tharakaraman K, Jayaraman A, Raman R, Viswanathan K, et al. 2013. Glycan receptor binding of the influenza A virus H7N9 hemagglutinin. *Cell* **153**: 1486–93.
47. Matrosovich M, Zhou N, Kawaoka Y, Webster R. 1999. The surface glycoproteins of H5 influenza viruses isolated from humans, chickens, and wild aquatic birds have distinguishable properties. *J Virol* **73**: 1146–55.
48. Banks J, Plowright L. 2003. Additional glycosylation at the receptor binding site of the hemagglutinin (HA) for H5 and H7 viruses may be an adaptation to poultry hosts, but does it influence pathogenicity? *Avian Dis* **47**: 942–50.
49. Imai M, Watanabe T, Hatta M, Das SC, et al. 2012. Experimental adaptation of an influenza H5 HA confers respiratory droplet transmission to a reassortant H5 HA/H1N1 virus in ferrets. *Nature* **486**: 420–8.
50. Herfst S, Schrauwen EJA, Linster M, Chutinimitkul S, et al. 2012. Airborne transmission of influenza A/H5N1 virus between ferrets. *Science* **336**: 1534–41.
51. Zhang W, Shi Y, Lu X, Shu Y, et al. 2013. An airborne transmissible avian influenza H5 hemagglutinin seen at the atomic level. *Science* **340**: 1463–7.
52. Wang W, Lu B, Zhou H, Suguitan AL, et al. 2010. Glycosylation at 158N of the hemagglutinin protein and receptor binding specificity synergistically affect the antigenicity and immunogenicity of a live attenuated H5N1 A/Vietnam/1203/2004 vaccine virus in ferrets. *J Virol* **84**: 6570–7.
53. Linster M, van Boheemen S, de Graaf M, Schrauwen EJA, et al. 2014. Identification, characterization, and natural selection of mutations driving airborne transmission of A/H5N1 virus. *Cell* **157**: 329–39.
54. Russell CA, Fonville JM, Brown AEX, Burke DF, et al. 2012. The potential for respiratory droplet-transmissible A/H5N1 influenza virus to evolve in a mammalian host. *Science* **336**: 1541–7.
55. Neumann G, Macken CA, Karasin AI, Fouchier RA, et al. 2012. Egyptian H5N1 influenza viruses-cause for concern? *PLoS Pathog* **8**: e1002932.
56. Maines TR, Chen L-M, Van Hoeven N, Tumpey TM, et al. 2011. Effect of receptor binding domain mutations on receptor binding and transmissibility of avian influenza H5N1 viruses. *Virology* **413**: 139–47.
57. Bullough PA, Hughson FM, Skehel JJ, Wiley DC. 1994. Structure of influenza haemagglutinin at the pH of membrane fusion. *Nature* **371**: 37–43.
58. Skehel JJ, Bayley PM, Brown EB, Martin SR, et al. 1982. Changes in the conformation of influenza virus hemagglutinin at the pH optimum of virus-mediated membrane fusion. *Proc Natl Acad Sci USA* **79**: 968–72.
59. Stevens J, Corper AL, Basler CF, Taubenberger JK, et al. 2004. Structure of the uncleaved human H1 hemagglutinin from the extinct 1918 influenza virus. *Science* **303**: 1866–70.
60. Steinhauer DA, Martin J, Lin YP, Wharton SA, et al. 1996. Studies using double mutants of the conformational transitions in influenza hemagglutinin required for its membrane fusion activity. *Proc Natl Acad Sci USA* **93**: 12873–8.
61. Russell RJ, Gamblin SJ, Haire LF, Stevens DJ, et al. 2004. H1 and H7 influenza haemagglutinin structures extend a structural classification of haemagglutinin subtypes. *Virology* **325**: 287–96.
62. Thoennes S, Li Z-N, Lee B-J, Langley WA, et al. 2008. Analysis of residues near the fusion peptide in the influenza hemagglutinin structure for roles in triggering membrane fusion. *Virology* **370**: 403–14.
63. Galloway SE, Reed ML, Russell CJ, Steinhauer DA. 2013. Influenza HA subtypes demonstrate divergent phenotypes for cleavage activation and pH of fusion: implications for host range and adaptation. *PLoS Pathog* **9**: e1003151.
64. Hay AJ, Wolstenholme AJ, Skehel JJ, Smith MH. 1985. The molecular basis of the specific anti-influenza action of amantadine. *EMBO J* **4**: 3021–4.
65. Harvey R, Martin ACR, Zambon M, Barclay WS. 2004. Restrictions to the adaptation of influenza A virus h5 hemagglutinin to the human host. *J Virol* **78**: 502–7.



66. DuBois RM, Zaraket H, Reddivari M, Heath RJ, et al. 2011. Acid stability of the hemagglutinin protein regulates H5N1 influenza virus pathogenicity. *PLoS Pathog* 7: e1002398.
67. Brass AL, Huang I-C, Benita Y, John SP, et al. 2009. The IFITM proteins mediate cellular resistance to influenza A H1N1 virus, West Nile virus, and dengue virus. *Cell* 139: 1243–54.
68. Everitt AR, Clare S, Pertel T, John SP, et al. 2012. IFITM3 restricts the morbidity and mortality associated with influenza. *Nature* 484: 519–23.
69. Lin YP, Wharton SA, Martín J, Skehel JJ, et al. 1997. Adaptation of egg-grown and transfectant influenza viruses for growth in mammalian cells: selection of hemagglutinin mutants with elevated pH of membrane fusion. *Virology* 233: 402–10.
70. Smeenk CA, Wright KE, Burns BF, Thaker AJ, et al. 1996. Mutations in the hemagglutinin and matrix genes of a virulent influenza virus variant, A/FM/1/47-MA, control different stages in pathogenesis. *Virus Res* 44: 79–95.
71. Keleta L, Ibricevic A, Bovin NV, Brody SL, et al. 2008. Experimental evolution of human influenza virus H3 hemagglutinin in the mouse lung identifies adaptive regions in HA1 and HA2. *J Virol* 82: 11599–608.
72. Koerner I, Matrosovich MN, Haller O, Staeheli P, et al. 2012. Altered receptor specificity and fusion activity of the haemagglutinin contribute to high virulence of a mouse-adapted influenza A virus. *J Gen Virol* 93: 970–9.
73. Roche B, Drake JM, Brown J, Stallknecht DE, et al. 2014. Adaptive evolution and environmental durability jointly structure phylogenetic patterns in avian influenza viruses. *PLoS Biol* 12: e1001931.
74. Handel A, Lebarbenchon C, Stallknecht D, Rohani P. 2014. Trade-offs between and within scales: environmental persistence and within-host fitness of avian influenza viruses. *Proc Biol Sci* 281: pii: 20133051.
75. Reed ML, Bridges OA, Seiler P, Kim JK, et al. 2010. The pH of activation of the hemagglutinin protein regulates H5N1 influenza virus pathogenicity and transmissibility in ducks. *J Virol* 84: 1527–35.
76. Robinson CM, Jesudhasan PR, Pfeiffer JK. 2014. Bacterial lipopolysaccharide binding enhances virion stability and promotes environmental fitness of an enteric virus. *Cell Host Microbe* 15: 36–46.
77. Sorrell EM, Wan H, Araya Y, Song H, et al. 2009. Minimal molecular constraints for respiratory droplet transmission of an avian-human H9N2 influenza A virus. *Proc Natl Acad Sci USA* 106: 7565–70.
78. Yen HL, Peiris JSM. 2012. Virology: bird flu in mammals. *Nature* 486: 332–3.
79. England RJ, Homer JJ, Knight LC, Eli SR. 1999. Nasal pH measurement: a reliable and repeatable parameter. *Clin Otolaryngol Allied Sci* 24: 67–8.
80. Washington N, Steele RJ, Jackson SJ, Bush D, et al. 2000. Determination of baseline human nasal pH and the effect of intranasally administered buffers. *Int J Pharm* 198: 139–46.
81. Elderfield RA, Watson SJ, Godlee A, Adamson WE, et al. 2014. Accumulation of human-adapting mutations during circulation of A (H1N1)pdm09 influenza in humans in the UK. *J Virol*, in press 88: 13269–83.
82. Cotter CR, Jin H, Chen Z. 2014. A single amino acid in the stalk region of the H1N1pdm influenza virus HA protein affects viral fusion, stability and infectivity. *PLoS Pathog* 10: e1003831.
83. Dorigatti I, Cauchemez S, Ferguson NM. 2013. Increased transmissibility explains the third wave of infection by the 2009 H1N1 pandemic virus in England. *Proc Natl Acad Sci USA* 110: 13422–7.
84. Cohen M, Zhang XQ, Senaati HP, Chen H-W, et al. 2013. Influenza A penetrates host mucus by cleaving sialic acids with neuraminidase. *Viral J* 10: 321.
85. Matrosovich MN, Matrosovich TY, Gray T, Roberts NA, et al. 2004. Neuraminidase is important for the initiation of influenza virus infection in human airway epithelium. *J Virol* 78: 12665–7.
86. Banks J, Speidel ES, Moore E, Plowright L, et al. 2001. Changes in the haemagglutinin and the neuraminidase genes prior to the emergence of highly pathogenic H7N1 avian influenza viruses in Italy. *Arch Virol* 146: 963–73.
87. Baigent S, McCauley J. 2001. Glycosylation of haemagglutinin and stalk-length of neuraminidase combine to regulate the growth of avian influenza viruses in tissue culture. *Virus Res* 79: 177–85.
88. Hoffmann TW, Munier S, Larcher T, Soubieux D, et al. 2012. Length variations in the NA stalk of an H7N1 influenza virus have opposite effects on viral excretion in chickens and ducks. *J Virol* 86: 584–8.
89. Castrucci MR, Kawaoka Y. 1993. Biologic importance of neuraminidase stalk length in influenza A virus. *J Virol* 67: 759–64.
90. Blumenkrantz D, Roberts KL, Shelton H, Lycett S, et al. 2013. The short stalk length of HPAI H5N1 influenza neuraminidase limits transmission of pandemic H1N1 virus in ferrets. *J Virol* 87: 10539–51.
91. Munier S, Larcher T, Cormier-Aline F, Soubieux D, et al. 2010. A genetically engineered waterfowl influenza virus with a deletion in the stalk of the neuraminidase has increased virulence for chickens. *J Virol* 84: 940–52.
92. Matsuoka Y, Swayne DE, Thomas C, Rameix-Welti M-A, et al. 2009. Neuraminidase stalk length and additional glycosylation of the hemagglutinin influence the virulence of influenza H5N1 viruses for mice. *J Virol* 83: 4704–8.
93. Zhou H, Yu Z, Hu Y, Tu J, et al. 2009. The special neuraminidase stalk-motif responsible for increased virulence and pathogenesis of H5N1 influenza A virus. *PLoS One* 4: e6277.
94. Sun Y, Tan Y, Wei K, Sun H, et al. 2013. Amino acid 316 of hemagglutinin and the neuraminidase stalk length influence virulence of H9N2 influenza virus in chickens and mice. *J Virol* 87: 2963–8.
95. Sorrell EM, Song H, Pena L, Perez DR. 2010. A 27-amino-acid deletion in the neuraminidase stalk supports replication of an avian H2N2 influenza A virus in the respiratory tract of chickens. *J Virol* 84: 11831–40.
96. Chutinimitkul S, van Riel D, Munster VJ, van den Brand JMA, et al. 2010. In vitro assessment of attachment pattern and replication efficiency of H5N1 influenza A viruses with altered receptor specificity. *J Virol* 84: 6825–33.
97. Shelton H, Roberts KL, Molesti E, Temperton N, et al. 2013. Mutations in hemagglutinin that affect receptor binding and pH stability increase replication of a PR8 influenza virus with H5 HA in the upper respiratory tract of ferrets and may contribute to transmissibility. *J Gen Virol* 94: 1220–9.
98. Li J, Zu Dohna H, Cardona CJ, Miller J, et al. 2011. Emergence and genetic variation of neuraminidase stalk deletions in avian influenza viruses. *PLoS One* 6: e14722.
99. Cox RJ, Madhun AS, Hauge S, Sjursen H, et al. 2009. A phase I clinical trial of a PER.C6 cell grown influenza H7 virus vaccine. *Vaccine* 27: 1889–97.
100. Karron RA, Talaat K, Luke C, Callahan K, et al. 2009. Evaluation of two live attenuated cold-adapted H5N1 influenza virus vaccines in healthy adults. *Vaccine* 27: 4953–60.
101. Chan W, Zhou H, Kemble G, Jin H. 2008. The cold adapted and temperature sensitive influenza A/Ann Arbor/6/60 virus, the master donor virus for live attenuated influenza vaccines, has multiple defects in replication at the restrictive temperature. *Virology* 380: 304–11.
102. Krenn BM, Egorov A, Romanovskaya-Romanko E, Wolschek M, et al. 2011. Single HA2 mutation increases the infectivity and immunogenicity of a live attenuated H5N1 intranasal influenza vaccine candidate lacking NS1. *PLoS One* 6: e18577.
103. Luke CJ, Subbarao K. 2006. Vaccines for pandemic influenza. *Emerg Infect Dis* 12: 66–72.
104. Center for Disease Control and Prevention. 2012. *Influenza Risk Assessment Tool (IRAT)*. <http://www.cdc.gov/flu/pandemic-resources/tools/risk-assessment.htm>.
105. Gabbard JD, Dlugolenski D, Van Riel D, Marshall N, et al. 2014. Novel H7N9 influenza virus shows low infectious dose, high growth rate, and efficient contact transmission in the guinea pig model. *J Virol* 88: 1502–12.